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Regulation and Function of Cytokines That Predict Prostate Cancer Metastasis

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

We identified biomarkers that help distinguish aggressive disease from those that do not progress following prostatectomy. Specifically, CX3CL1 and IL15 were identified to be downregulated in subjects that developed recurrent prostate cancer. Conversely, CCL4 was upregulated patients with recurrent disease. The role of Stat3 activation and p53 intracellular signaling downstream of these cytokines commonly seem to differentialy regulate invasion and sensitivity adhesion dependent survival. Further, these two cytokines similarly reduced adhesion of LNCaP cells to collagen I. However, sensitivity to aniokis was dramatically induced in the same cells by CX3CL1 and IL15. This data support the clinical observation of recurrent free subjects having greater expression of CX3CL1 and IL15. Further, these factors may even serve as anti-metastatic mediators, despite their limited effects on tumor cell proliferation.

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Progress Report: Regulation and Function of Cytokines That Predict Prostate Cancer Metastasis

a. **INTRODUCTION**

There is a large disparity between the number of newly diagnosed cases of prostate cancer in the United States every year and the number of men who die of the disease. The 30,000 deaths annually in the US caused by prostate cancer are almost entirely due to metastatic progression [1]. As a consequence, even though prostate cancer is the second leading cause of cancer related mortality in men in the United States, there is an ongoing concern that as a medical community we are over diagnosing, and hence over treating, the disease. *Yet, patients at high-risk for metastatic progression are unfortunately treated too late*. The challenge has been to determine up-front which patients harbor high-risk disease requiring aggressive/curative therapy and which patients harbor indolent disease that could be managed with active surveillance. The issue is an important one given the potential for attempts at local curative therapy (whether it be surgery, radiation or cryotherapy) to subject the patient to both short-term and long-term morbidity. Currently clinicians rely on a combinatorial assessment of the pre-treatment PSA value, clinical tumor stage, and biopsy-Gleason score to risk stratify patients. These methods are unable to distinguish 80% of the patients that may not have any clinical consequences from the prostate cancer [2]. Next, following local curative therapy the issue of requirement and timing for second line adjuvant therapy becomes increasing important. However, treatment of cancers prior to metastatic progression with conventional chemotherapy has shown promise of late [3-5]. It is critical to accurately determine the appropriate candidates for such adjuvant therapy given the potential for decreased quality of life and added morbidity associated with chemotherapy treatment, especially since the majority (65%) of patients remain disease free after prostatectomy. Since men experiencing PSA recurrence following surgical treatment suggest metastatic spread of the disease, better forms of early detection and risk stratification would support targeted use of adjuvant therapies [6]. Similar to the clinical stratification described for patients prior to primary treatment for prostate cancer, pathologic risk of biochemical recurrence is performed following primary treatment. One of the most commonly used nomogram for post-operative predictions has been described by Kattan and colleagues [7, 8]. Multiple criteria that include the pre-treatment PSA, prostate capsule invasion, pathologic Gleason score, surgical margin status, seminal vesicle involvement, and lymph node involvement for predicting post-operative biochemical recurrence [7, 8]. However, a model of such clinical/pathologic parameters is limited (particularly at the level of sensitivity) by the fact we do not know all the predictive factors.

Chemokines, cytokines, and growth factors in the tumor microenvironment regulate the fate of tumor progression [9, 10]. We hypothesized that tissue chemokines can be strong biomarker candidates for distinguishing patients with high risk for biochemical recurrence or metastatic progression of prostate cancer. CX3CL1 exhibited the best prediction ability ($P < 0.0001$) followed by CCL4 ($P < 0.001$) and IL-15 ($P = 0.003$). The proportional hazard assumption was tested with scaled Schoenfeld residuals [11]. There was no evidence of violation as the chi-square tests for trend were not significant for any of the seven variables (surgical margin status, seminal vesicle involvement, Gleason Score, pre-operative PSA, CCL4, and CX3CL1, and IL-15; P values ranging 0.46 to 0.90). The same two chemokines, CCL4 ($P=0.040$) and CX3CL1 ($P<0.0001$) were significant factors. In addition, pre-operative PSA ($P= 0.0025$) and surgical margin ($P= 0.023$) were significant [12]. We described a strong predictive ability of differentially expressed chemokines in a nested case-control study of prostate cancer patients that develop biochemical recurrence or lead recurrent-free lives following prostatectomy. The goal of this proposal is to determine the biologic role of these potentially clinically relevant chemokines in prostate cancer progression.

In this past year, we focused on the role of stromally derived IL15 and CX3CL1 - the two cytokines that are downregulated in PCa patients with recurrent disease. However, based on our past findings on the recruitment of bone marrow derived cells (BMDCs) and the rapid prostatic proliferation during CRPC-associated regrowth. Tissue remodeling, and cancer progression are generally associated with the recruitment of BMDCs [13]. Co-expression of prostate markers with BMDCs suggested that these recruited cells were also incorporated into the prostate epithelia [14]. We further identified MSCs fusing with prostatic epithelia. Interestingly, the chemokine,

CCL4, up regulated in patients with biochemical recurrence, recruits MSC. CX3CL1, down regulated in the recurrent population, is critical to the communication with MSC in eliciting anti-tumor activity. However, the goals this year was to characterize the paracrine (not systemic) role of IL15 and CX3CL1 on prostatic epithelia.

b. **BODY**

The data described here is work done as a result of this DOD award. The focus of **Aim 1** was to determine the effect of the differentially expressed chemokines on prostate cancer cells. Our knowledge of the importance of the tumor microenvironment in prostate cancer metastatic progression is addressed in **Aim 2** of the proposal. In the past progress reports we discussed progress on the biologic role of CCL4 in prostate cancer progression through the development of in vitro and in vivo models. Further, the expression of CX3CL1 and IL15 by prostatic fibroblastic cells reduces prostatic epithelial cell viability in suspension cultures through an apoptotic mechanism. In the past year we have made significant progress in better understanding of the mechanism of action supporting the previously reported observations.

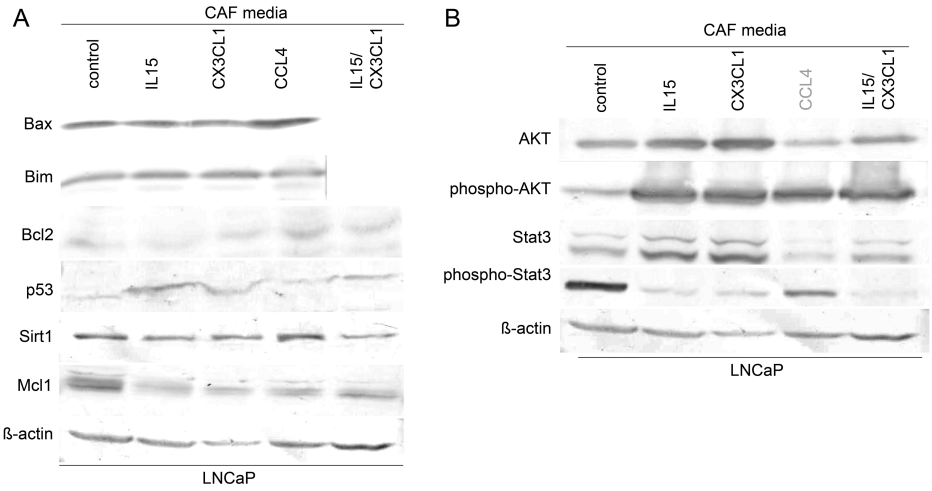

Figure 1. Prostatic fibroblasts were transfected with CX3CL1 and IL15 expression constructs followed by blasticidine selection. A GFP vector was introduced one fifth of either CX3CL1 or IL15 constructs to monitor transfection efficiency (upper panels). RT PCR for CX3CL and IL15 expression in transfected (T) cells were compared to GFP only transfected control (C, lower panels).

The goal of this proposal was to determine the biologic rational for the three chemokines: CX3CL1, CCL4, and IL-15, that helped distinguish prostate cancer patients that developed recurrent disease [12]. We found that in fact, the cytokines originally found to be downregulated in patients with recurrent free disease – CX3CL1 and IL-15 – promoted apoptosis in LNCaP cells associated with the elevation of p53 (**Figure 1**). Whereas, CLL4 expression – associated with recurrent disease – seemed to prevent apoptosis by dowregulating p53. The differential regulation of p53 by the cytokines, resulted in an inverse change in Bcl2 (antiapoptotic protein) expression. However, Bax, a proapoptotic pore protein, did not seem to follow the prescribed coupling role with p53 to

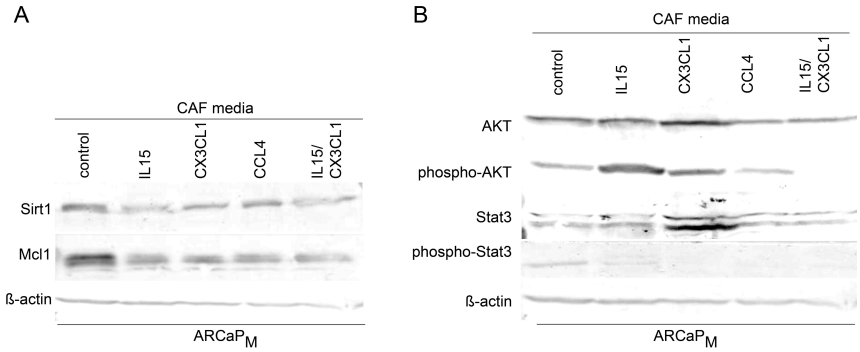

Figure 2. Addition of stromal conditioned media expressing CX3CL1 and IL15 reduces the proliferation of LNCaP and BPH1 cells. Recombinant cytokines expressed in 72hr conditioned media was added to prostatic cancer (LNCaP) and non-tumorigenic (BPH1) cells. Proliferation was measured by sequential counting over a 96 h period. The blue line indicatesconditioned media from GFP expressing control prostatic fibroblasts.

regulate apoptosis in the LNCaP epithelia in response to the respective cytokines. In fact, CCL2 seemed to upregulate Bax, although reduced apoptosis was observed with this cytokine (**Figure 1**). Similarly, Sirt1 (potent inhibitor of surviving) followed the expression pattern of Bax in a counterintuitive manner to that of apoptotic pattern observed with each of the three cytokines. Interestingly, Bim expression remained unchanged in the presence of the cytokines. Apart from the expected role of Bcl2, the other conventional apoptotic signaling pathway components do not seem to follow the biologic apoptotic observation. Examining the AKT and Stat3 signaling pathways directed us toward a novel signaling pathway involving p53 and Stat3 activation. We found that CX3CL1 and IL-15 downregualted Stat3 activation, whereas CCL4 did not appreciably downregulate Stat3 phosphorylation. Thus, we feel Stat3 downregulation limits p53 expression that in turn affects the cellular response to adhesion independent survival. Interestingly AKT was reproducibly upregulated in both LNCaP and ARCaP$_M$ cells by CX3CL1- and IL-15-containing CAF conditioned media treatment (**Figure 2**). Normally AKT activation is associated with cell survival pathways. However, the roleof AKT in cytokine effects on aniokis is less clear. Here, we show for the first time that Stat3 and p53 support cytokine-mediated aniokis resistance.

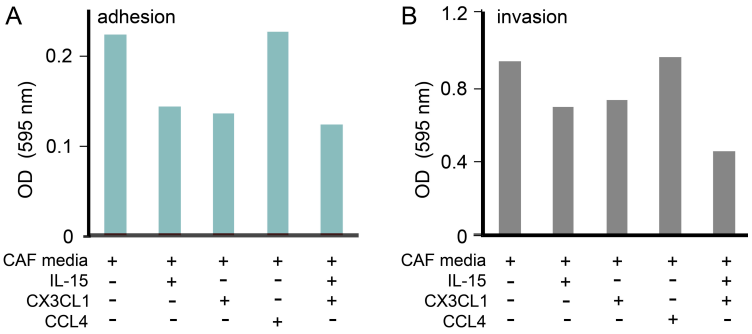

Figure 3. LNCaP cell motility is significantly diminished by CX3CL1 and IL15, based on scratch assay results. The motility of BPH1 cells was not affected similarly by the cytokines. (Similar results with transwell assays is not shown.)

Interestingly, the pattern of adherence independent survival paralleled prostate epithelial cell invasion. We found that CCL4, the cytokine associated with recurrent disease, promoted prostate epithelial cell invasion. Conversely, CX3CL1 and IL-15 expression by CAF cells limited LNCaP cell invasion (**Figure 3**). More significantly, the prevention of invasion was cooperative when IL-15 and CX3CL1 was combined, compared to the cytokines alone.

A number of attempts were made in the development of in vivo models to test these exciting in vitro studies. Unfortunately, host mice use for these studies we not able to support the expansion of the tissue recombinants. Despite our extensive experience in developing tissue recombinants, the tissue recombinants with ARCaPM epithelia (we had never attempted in the past) have not expanded in Nude or SCID mice. We have discovered the rational for this observation to be due to the level of NK cells in these host lines. We are currently performing the proposed studies in Beige SCID mice that have reduced NK cell populations. Despite the depletion of funds for this proposal, we are continuing with all the proposed studies, for the development of a strong body of biologic support for the use of CX3CL1, IL15 and CCL4 as a biomarker and likely targets for therapeutic intervention.

c. **KEY RESEARCH ACCOMPLISHMENTS**

- We demonstrated that both CX3CL1 and IL15 paracrine signaling can reduce both the proliferation of both tumorigenic and non-tumorigenic prostatic epithelia.
- CCL4 expression by CAF do not seem to affect proliferation or invasion of prostatic epithelia.
- We demonstrated that both CX3CL1 and IL15 paracrine signaling has little effect on the motility of non-tumorigeneic BPH1 cells, but interestingly reduced tumorigenic LNCaP cell motility.
- We identified that both CX3CL1 and IL15 expression caused increased LNCaP sensitivity to aniokis, not BPH1 cells.
- Identified Stat3 and p53 signaling as primary mediators of aniokis resistance by CLL4 and conversely, CX3CL1 and IL15 mediated sensitivity to aniokis.

d. **REPORTABLE OUTCOMES**

Research

Publication

none.

Awards received based on work supported by this grant

Prostate Cancer Foundation 09/01/12-08/31/14

Isaacs/Karp/Bhowmick (PI) $1,000,000 direct cost

First-in-Man Clinical Studies of Mesenchymal Stem Cell Based Therapy for Prostate Cancer

The proposal tests the hypothesis that allogeneic human bone marrow-derived MSCs can be loaded with microparticles containing a drug so that when infused, they selectively deliver drug to metastatic sites of prostate cancer, thus sparing host toxicity. It involves the testing of the methodology in mouse models and performing phase zero studies in men with prostate cancer.

Products

CDNA construct, cell lines, and animal models developed

- Generated CX3CL1 over expressing human prostatic fibroblastic cells
- Generated IL15 over expressing human prostatic fibroblastic cells
- Generated CCL4 over expressing human prostatic fibroblastic cells
- Model system for spontaneous prostate cancer metastasis through tissue recombination of CCL4 expressing human stromal fibroblastic cells and non-metastatic LNCaP epithelia

e. **CONCLUSION**

The identification of CX3CL1, IL-15, and CCL4 as differentially expressed chemokines used to predict biochemical recurrence following prostatectomy supported the proposed studies where by CX3CL1 and IL-5 expression was associated with recurrent-free survival, where as CCL4 expression was associated with recurrence [12]. Thus the direct effects of CCL4 of cancer epithelia of varying metastatic potential were presented previously. In this report we focused on the biologic role of CX3CL1 and IL15 on prostate epithelia. The Stat3 and p53 signaling pathway was identified as a probable mechanism for survival of PCa cells in circulation and tissue invasion. Overwhelming data in this series of experiments suggest that these cytokines are not only downregulated in recurrent PCa subjects, but may in fact be anti-metastatic factors expressed by the stromal microenvironment. Previous studies have suggested the pro-tumorigenic properties of the tumor associated stromal fibroblastic cells. However, our data presented here support that the stroma can have an inhibitory role in metastatic progression. The elevated expression of extracellular matrix by carcinoma associated fibroblasts has been associated with restricting metastatic PCa progression [15]. However, here we show that specific cytokines can also have a restrictive role in metastatic progression. Importantly, CX3CL1 and IL15 had little effect of tumor cell proliferation, but rather the characteristics needed for distant metastatic progression. The on going xenografting experiments will tell if these results hold true in vivo.

The Prostate Cancer Foundation grant awarded due to the results generated due to the support of in this DOD application will enable “first in man” neo-adjuvant studies in PCa patients with locally advanced disease.

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